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14. ABSTRACT
We are testing, in prospective studies from Hopkins (Brady) and Harvard (PHS, HPFS), whether the combination of telomere length variability in prostate cancer cells and short telomere length in cancer-associated stromal cells is an independent prognostic indicator of poor prostate cancer outcome. In Year 4, we determined that the criteria we defined in Year 3 support that we now have a sufficiently optimized protocol for semi-automated slide scanning and multi-channel acquisition of fluorescent images using the TissueFAXS Plus microscopy workstation and TissueFAXS 4.0 software (Tissue Gnostics). We documented within- and between-operator reliability in determination of telomere length is sufficient for cancer and cancer associated stromal cells, and determined that some within-operator variability is due to biological variability rather than method or operator variability. We will disseminate the method and the reliability via publication (in preparation). To make this "test" viable as a clinical tool, we continue to make refinements to achieve full automation of the method to distinguish among cell types to include/exclude for the telomere biomarker. We have begun Tasks 5, 6, and 7.

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INTRODUCTION: Currently used clinico-pathologic prognostic factors are imperfect predictors of outcome in the men with clinically localized prostate cancer, the majority of men diagnosed today. Thus, tissue-based biomarkers that significantly enhance the predictive power are urgently needed to improve treatment and surveillance decision-making for these men. To address this pressing clinical need, we have assembled a multidisciplinary prostate cancer research team from Johns Hopkins and Harvard to validate and optimize a novel tissue biomarker of prognosis for men with clinically localized prostate cancer that we recently identified – telomere length variability in prostate cancer cells combined with short telomere length in cancer-associated stromal cells. In our prior work, men with this combination had a substantially higher risk of dying of their prostate cancer compared with men without this combination. Equally importantly, men without this combination rarely died of their prostate cancer over 10 years. Key next steps to realize the great translational potential of telomere length as an independent prognostic tissue biomarker are optimized biomarker assessment and validation. Thus, our aims are to: 1) Optimize the method for assessing telomere length by FISH using a high-throughput approach to yield a test feasible for the clinical setting. 2) Validate our compelling findings in two other cohort studies on prostate cancer outcomes: a) men surgically treated and followed for lethal prostate cancer; and b) men surgically treated and followed for prostate cancer recurrence. 3) Determine optimal biomarker cutpoints for prognosis.

BODY: This work is being performed collaboratively by two institutions: Johns Hopkins Bloomberg School of Public Health and Harvard School of Public Health. This progress report covers Year 4 ("Progress in Year 4"). For completeness and context, we also provide the progress we reported for Years 1-3 ("Progress in Years 1, 2, and 3").

Progress in Years 1, 2, and 3:
In Year 1, we obtained all required IRB approvals for both the Brady prostate cancer recurrence Study (Hopkins), the Physicians' Health Study (PHS, Harvard) and the Health Professionals Follow-up Study (HPFS, Harvard), including from the DOD IRB (**Task 1 completed**). Drs. Platz and De Marzo previously created the Brady prostate cancer recurrence nested case-control study (Brady study; in part with prior DOD funding to Dr. Platz at Hopkins) and associated tissue microarrays (TMAs). This TMA set is now part of the Prostate Cancer Biorepository Network (PCBN). For equitable use and tracking purposes, during Year 1, we applied for access to these TMAs and received approval from the PCBN. For the Brady study, we pulled the recurrence TMAs (N=16 TMAs, which includes 524 cases and 524 controls) and cut and mounted the sections (**Task 4b completed**) for staining for telomere-specific FISH, cytokeratin 903 immunofluorescence, and for DAPI.

The PHS and HPFS are existing cohort studies, and TMAs have been constructed for those participants who underwent a radical prostatectomy with other funds, including previous DOD funding (to Dr. Mucci at Harvard). In Year 2, we received sections from the 6 PHS TMAs and 3 HPFS TMAs that had not yet been constructed when we conducted our prior work on the telomere biomarker and that served as the preliminary data for the proposal for the current study (**Task 4a completed**).

In Year 1, the Johns Hopkins investigators purchased a new state-of-the art fluorescence slide scanner and associated image analysis software from Tissue Gnostics using donor funds (**Task 2a completed**). This new system uses the TissueFAXS Plus (Tissue Gnostics, Vienna, Austria) microscopy workstation for slide-based cytometry of tissue sections and TMAs.

In Year 2, we developed and began to optimize protocols for fully automated slide scanning and multi-channel acquisition of fluorescent images using a 40X oil objective. We demonstrated the ability to image prostate tissues that have been fluorescently co-stained with a Cy3-labelled telomere specific peptide nucleic acid probe (red), a prostate basal cell-specific anti-cytokeratin primary antibody and detected with a fluorescent secondary antibody conjugated to Alexa Fluor 488 (green), and counterstained with DAPI to detect the nuclei (blue). In addition, we utilized the Tissue Gnostics image analysis software to begin to automate the segmentation of individual cell nuclei and telomere FISH signals (**Task 2b completed**).

In Year 3, we further refined and neared completion of developing a fully optimized and automated imaging system for single cell telomere length analyses. First, we documented our ability to reliably scan and analyze an entire prostate TMA. Next, we imaged an entire TMA at 40X magnification (sufficient for image analysis) by collecting images on 4 different channels (DAPI, Cy2, Cy3, Cy5), followed by segmentation of the images into specific TMA spots. Next, we demonstrated our ability to detect individual cells of specific cell types, and perform image analysis for these specific cells types. This step was significantly enhanced by adding the prostate epithelial-specific marker NKX3.1 (detected in the Cy5 channel) to our antibody panel in the multiplex staining. From captured images, we developed (i) nuclear segmentation parameters based on the DAPI and Cy5 channels that specifically identify cancer cell nuclei and (ii) a specialized algorithm to detect the telomeres within the nuclei. In addition, we developed nuclear segmentation parameters based on the DAPI channel and lack of signal in the Cy5 channel that specifically identified cancer-associated stromal nuclei and the specialized algorithm to detect the telomeres within the nuclei. Finally, we demonstrated the ability to quantitate the telomere signals on a per nucleus basis in both the cancer and cancer-associated stromal (CAS) cells. Telomere-specific FISH signals are linearly proportional to telomere length and thus, telomere length can be quantified via digital image analysis. Telomere length, on a per cell basis, is determined as the ratio of the total intensity of telomeric signals in each cell to the total intensity of the DAPI stained nuclear DNA signal in the same cell.

In Year 3, the Hopkins and Harvard investigators met in person to review and summarize progress and determined that Task 3 (*Test the automated method of telomere length determination for precision and validity by rerunning the automated method (precision) and by comparing to the nonautomated method (validity)*), which would have involved rescanning the original 5 HPFS TMA sections to compare the automated method to the nonautomated method of telomere length determination for precision and validity, would not be informative. During optimization of the method, we

concluded that the process of capturing the TMA images adversely affects the fluorescence of both the Cy3 telomere signals, as well as the DAPI DNA signals, in nonsystematic ways, due to photobleaching. We decided that we will instead perform Task 3 using 2 (of 7) adjacent sections of the test TMA provided by Harvard (contains HPFS prostate cancer and normal adjacent tissue). We also developed a set of criteria we said we would use to decide whether the currently established automated method is sufficiently optimized to move forward with Tasks 5, 6, and 7 in Year 4.

Progress in Year 4:
At the beginning of Year 4, we requested and received the Brady TMAs (**Task 4**). We had previously requested and received the 2 additional new HPFS TMAs and PHS TMAs.

We evaluated the 6 criteria we developed in Year 3 for determining whether our currently established automated method is sufficiently optimized to move forward with Tasks 5, 6, and 7. Using in-house TMAs and sections of a test TMA provided by Harvard (constructed using the same prostate cancer tissue repository as in the HPFS) we determined that: 1) FISH/immunofluorescence appropriately labels the telomeres, centromeres, and specific cell types (basal epithelial, luminal epithelial, cancer); 2) 7 in-focus images for each channel (DAPI, Cy2, Cy3, Cy5) are captured without signal intensity saturation and/or overexposure; 3) individual TMA spots can be clearly segmented; 4) labeling and quality of the image capture is sufficient for the Tissue Gnostics software platform to perform image analysis of telomere signal intensity for individual cells and types of cells; 5) software parameters (e.g. nuclei size, nuclei compactness, sensitivity threshold of dot detection, etc.) are optimized for the different signal intensities of cancer cells (dim) versus cancer-associated stromal cells (strong). Using sections of the test TMA from Harvard, 6) we determined that 1-5 above produce reproducible telomere length values. To do so, we used two sections of the test TMA. We stained and imaged the sections on different occasions and independently performed image analysis (Run 1 [test TMA section 1], Run 2 [test TMA section 2]). Two operators performed the work, one highly experienced (Operator 1) and one newly trained (Operator 2). In the tables below, “CAN” refers to cancer cells and “CAS” refers to cancer-associated stromal cells. The test TMA contained 29 spots for 14 individuals.

With respect to within-operator reliability in the determination of telomere length (Table 1), sources of variability are: different TMA sections (not necessarily the same cells or parts of the same cells being evaluated), staining variability, imaging variability, and within-operator variability. Note that the two operators used the same images. As expected, telomere length was substantially shorter in cancer than in cancer-associated stromal cells. The median % difference in cancer cells between the two runs was 21.5% and 31.8% for the two operators. Note that the magnitude of values for cancer cells is small, so any variability in the assessment of telomere length between the two runs appears as a larger median % difference. The median % difference for cancer-associated stromal cells was 15.5% and 14.2% for the two operators. Given that some of the variability between the runs is due to biological variability and staining/imaging variability, we are pleased with the within-operator reliability and note that the two

operators, who have different extents of experience with the method, had similar reliabilities for cancer-associated stromal cells, and given the scaling of the telomere length in cancer cells, not dissimilar reliabilities for cancer cells.

Table 1. Within-operator reliability in the determination of telomere length using the optimized, semi-automated method, separately by an experienced (Operator 1) and new (Operator 2) operators

	CAN					CAS				
Telomere Ratio	N_{pairs}	Median Run 1 [test TMA section 1]	Median Run 2 [test TMA section 2]	Median Difference *	Median % difference	N_{pairs}	Median Run 1 [test TMA section 1]	Median Run 2 [test TMA section 2]	Median Difference *	Median % difference
Median										
Operator 1	14	1.6	1.4	0.2	**21.5**	14	16.1	20.8	-3.45	**15.5**
Operator 2	14	1.6	1.5	-0.09	**31.8**	14	16.0	17.0	-0.59	**14.2**

*Difference is based on Run 1 subtract Run 2

With respect to between-operator reliability in the determination of telomere length (Table 2), the source of variability in this analysis is between operators. Note that the two operators both reviewed the images from test TMA section 1 and both reviewed the images from test TMA section 2. We found that the two operators, one experienced and one newly trained, were very consistent in their determination of telomere length when using the same test TMA. For both cancer and cancer-associated stromal cells, the median % difference was less than 10% for both operators in their evaluation of both test TMA sections.

Table 2. Between-operator reliability in the determination of telomere length using the optimized, semi-automated method, separately in two runs

	CAN					CAS				
Telomere Ratio	N_{pairs}	Median Operator 1	Median Operator 2	Median Difference *	Median % difference	N_{pairs}	Median Operator 1	Median Operator 2	Median Difference *	Median % difference
Median										
Run 1 [test TMA section 1]	14	1.6	1.6	-0.01	**5.6**	14	16.1	16.0	0.24	**2.7**
Run 2 [test TMA section 2]	14	1.4	1.5	-0.02	**7.8**	14	20.8	17.0	0.40	**6.4**

*Difference is based on Operator 1 – Operator 2 (separately by run)

Next, we compared telomere length in 2 TMA spots on the same test TMA section (staining and imaging in the same run) from the same man. We used data from operator

1, run 1 (test TMA section1). We observed large TMA spot-to-spot variability in cancer cells (45.9%). Note that this difference is based on a magnitude of values for cancer cells that is small, such that any variability in the determination of telomere length between the two TMA spots appears as a larger median % difference. Yet, this variability is greater than that observed in Table 1 (in which variability included biological, staining and imaging, and within-operator variability), suggesting that variability due to the method (staining/imaging) is low. In cancer-associated stromal cells, we observed a small median % difference (4.1%) between 2 TMA spots from the same man using the same TMA section. Note that in the cancer, we expected more biological variability in telomere length between two TMAs spots sampled from different horizontal positions in the tumor from the same man than in two vertical looks at the same TMA spot in the same cancer from the same man. For cancer-associated stromal cells, we expected less biological variability than in the cancer, and expected stromal cell telomere length from horizontal (2 different TMA spots – 4.1%, Table 3) and vertical (2 different sections of the same TMA spot – ~15%, Table 1) looks were would be less variable than in the cancer.

Table 3. Assessment of variability in telomere length due to biological variability and within-operator variability (rather than staining variability, imaging variability, and between-operator variability)*

	CAN					CAS				
Telomere Ratio	N_{pairs}	Median TMA spot 1	Median TMA spot 2	Median Difference *	Median % difference	N_{pairs}	Median TMA spot 1	Median TMA spot 2	Median Difference *	Median % difference
Median Run 1 (test TMA section 1)	9	1.1	2.1	-0.2	**45.9**	9	15.9	15.3	0.3	**4.1**

*Operator 1. Some men had >2 spots. Selected the 2 spots with the most number of cells evaluated and only included men who had both CAN and CAS evaluable.

The Hopkins and Harvard investigators again met in person at the annual Prostate Cancer Foundation Retreat (no cost to DOD for travel) to review and summarize Year 4 progress for this progress report. After reviewing all 6 of the criteria we established in Year 3, including the above reliability data, our team members are in agreement that the methods are sufficiently valid, robust and reproducible. At the end of Year 4, the method is semi-automated and we have begun Tasks 5, 6, and 7 with one refinement described below. We are completing a draft manuscript describing the method and its reliability for publication. The manuscript provides sufficient technical detail for others in the field to implement the method.

To make this “test” viable as a clinical tool, we have as a goal full automation. Thus, we continue to refine some aspects of the method. For example, during our analysis of the test TMA, we encountered a subset of cases in which we observed a heterogeneous staining pattern of the prostate epithelial-specific marker NKX3.1 (detected in the Cy5 channel), which we use to easily and accurately detect cancer cells from the

surrounding cancer-associated stromal cells. To further refine our assay, we have now included another prostate epithelial-specific marker FOXA1 (also detected in the Cy5 channel). As shown in Figure 1, we compared adjacent sections of a prostate tumor block and observed a significant signal increase when both antibodies are in included in the multiplex staining, as opposed to just a single antibody alone. Again, we are using both NKX3.1 and FOXA1 to identify cancer cells as distinct from surrounding cancer-associated stromal cells.

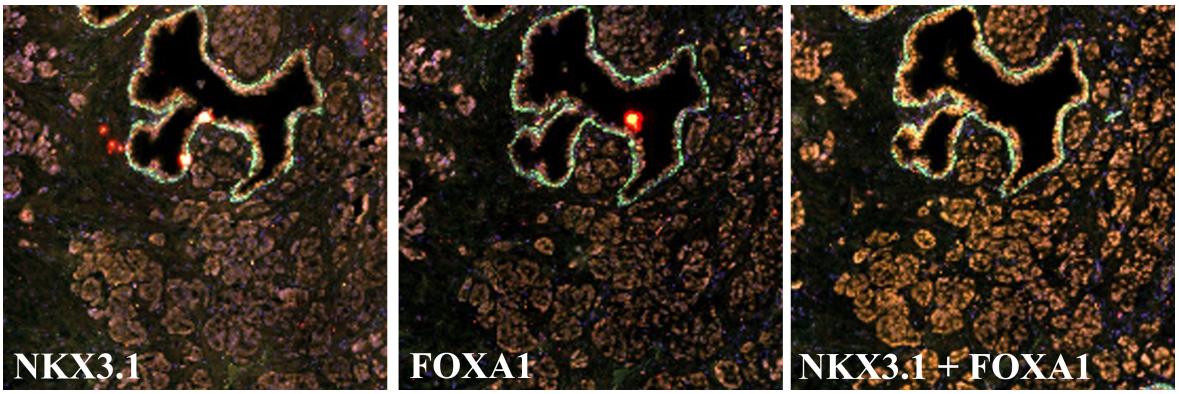

Figure 1. Telomere-specific FISH and immunostaining in a prostate tumor section. These representative images contains both normal prostate glands (basal and luminal cells) embedded in prostatic stroma and an area of tumor cells/glands in areas of cancer-associated stroma. DAPI (blue), telomeric DNA (red), and basal cells are demarcated with a basal cell-specific cytokeratin antibody (green). Immunostaining in orange is for NKX3.1 alone (left panel), FOXA1 alone (middle panel), or NKX3.1+FOXA1 in combination (right panel) highlights cancer cells and normal luminal cells. Note: at this power, the telomeric DNA (red) is not visible. Each panel consists of 56 images taken at 40X magnification that are stitched together.

Next steps and plans for Year 5:
We are currently staining and analyzing the 2 additional HPFS TMAs, the PHS TMAs, and the Brady TMAs with the newly refined multiplex FISH and antibody panel and moving toward completion of Tasks 5, 6, and 7.

We will submit the manuscript describing the method and its reliability as a way to disseminate the method to the research community.

We will continue to work toward full automation of the method for clinical use. For example, we are currently testing methods to automatically exclude lymphocytes (which using this method have particularly robust Cy5 intensity such that their inadvertent inclusion as a cancer-associated stromal cell could distort distributions). At the beginning of Year 5, we began testing the CD45 antibody, which is a general immune cell marker, versus a combination of CD3 and CD20 to rule in/out lymphocytes specifically automatically.

KEY RESEARCH ACCOMPLISHMENTS: None directly from this project to date (see above).

While we are pursuing the aims of the current DOD grant, our telomeres and prostate cancer team continues to conduct research addressing the aims and related questions using the data collected under our two prior DOD grants on telomeres (W81XWH-06-1-0052, W81XWH-05-1-0030).

Previously reported:

Joshu CE, Peskoe SB, Heaphy CM, Kenfield SA, Van Blarigan EL, Mucci LA, Giovannucci EL, Stampfer MJ, Yoon G, Lee TK, Hicks JL, De Marzo AM, Meeker AK, Platz EA. Prediagnostic Obesity and Physical Inactivity Are Associated with Shorter Telomere Length in Prostate Stromal Cells. Cancer Prev Res (Phila). 2015 Aug;8(8):737-42. doi: 10.1158/1940-6207.CAPR-15-0097. Epub 2015 May 19. PubMed PMID: 25990087; PubMed Central PMCID: PMC4526348.

Julin B, Shui I, Heaphy CM, Joshu CE, Meeker AK, Giovannucci E, De Vivo I, Platz EA. Circulating leukocyte telomere length and risk of overall and aggressive prostate cancer. Br J Cancer. 2015 Feb 17;112(4):769-76. doi: 10.1038/bjc.2014.640. Epub 2015 Jan 6. PubMed PMID: 25562437; PubMed Central PMCID: PMC4333493.

New to this Year 4 progress report:

Weber KA, Heaphy CM, Rohrmann S, Gonzalez B, Bienstock JL, Agurs-Collins TD, Platz EA, Meeker AK. Influence of In Utero Maternal and Neonate Factors on Cord Blood Leukocyte Telomere Length: Clues to the Racial Disparity in Prostate Cancer? Prostate Cancer, 2016. In press.

Heaphy CM, Zarinshenas R, Baena-Del Valle JA, Kulac I, Graham MK, Joshu CE, De Marzo AM, Platz EA, Meeker AK. Tissue-based telomere length measurements as a biomarker for individualized prostate cancer risk stratification and prognostication (poster). Annual Prostate Cancer Foundation Scientific Retreat. October 8-10, 2015, Washington, DC.

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